African Journal of Biotechnology Vol. 9 (16), pp. 2437-2441, 19 April, 2010
Available online at http://www.academicjournals.org/AJB
ISSN 1684–5315 © 2010 Academic Journals

Full Length Research Paper

In vitro evaluation of antioxidant activity of *Anisopus mannii* N.E. Br.

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Accepted 11 December, 2009

***Anisopus mannii* (Asclepiadaceae) –a plant widely used as an anti-diabetic agent in Northern Nigerian traditional medicine, was subjected to antioxidant evaluation. The crude methanol extract, *n*-butanol and ethyl acetate fractions were investigated for free radical scavenging activity of the 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH), total phenolic contents and reducing power assay. The results of the DPPH free radical scavenging at 250 µgml^{-1} indicated maximum antioxidant activity of 94.1, 94.3 and 88.7% for crude methanol extract, ethyl acetate and *n*-butanol fractions, respectively. The total phenolic content was higher in the ethyl acetate fraction (59.60 mg/g gallic acid equivalent GAE) than in the *n*-butanol fraction (56.40 mg/g GAE) and crude methanol extract (53.20 mg/g GAE). The reducing power of *n*-butanol fraction was 0.112 nm higher than those of ethyl acetate fraction (0.110 nm) and crude methanol extract (0.106 nm). The antioxidant activities of the crude extract/fractions and the standards at maximum concentration of 250 µgml^{-1} decreased in the order: ascorbic acid> ethyl acetate fraction> crude methanol extract> Gallic acid> *n*-butanol fraction, but there was no significant difference ($p < 0.05$) between them. The phytochemical screening revealed the presence of flavonoids, tannins and saponins. This suggests a potential utility of the plant as a source of phenolic antioxidants and may provide leads in the ongoing search for natural antioxidants from Nigerian medicinal plants to be used in treating diseases related to free radical reactions.**

Key words: *Anisopus mannii*, antioxidants, 1, 1-diphenyl-2-picrylhydrazyl radical, reductive potential, total phenolic contents.

INTRODUCTION

Reactive oxygen species (ROS) which include free radicals such as superoxide anion radicals (O^{-}_{2}), hydroxyl radicals ($OH^{\bullet}$) and non free radical species such as H_2O_2 and singlet oxygen ($^1O_2$) are various forms of activated oxygen generated in the body (Yildirim et al., 2000; Gulcin et al., 2002). They have attracted increasing attention because of their role in cellular injury and ageing process (Lai et al., 2000). Under normal circumstances, the free radicals and ROS can be removed by the body's natural antioxidant defense, e.g glutathione peroxidase, catalase and superoxide dismutase (Aruoma, 1994). However, overproduction of ROS arising from either the mitochondrial electron transport chain or excessive stimulation of NAD (P)H, or from exposure to environmental pollutants, cigarette smoke, UV-rays, radiation and toxic chemicals (Valko et al., 2006) results in a weakened body defense system, hence creating the need to provide the body with a constant supply of phytochemicals through dietary supplementation. Antioxidants have the ability to prevent, delay or ameliorate many of the effects of free radicals.

Plants constitute an important source of active natural products which differ widely in terms of structure and biological properties. Several plants and vegetables used in traditional medicine can provide diverse secondary metabolites with antioxidant potentials most of which are isolated phenolic compounds (Ramarathnam et al., 1997;

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Odukoya et al., 2005). Phenolic natural products such as flavonoids are of particular interest because of their antioxidant activity through scavenging oxygen radicals. The antioxidant capacity of these phenolic compounds is mainly due to their redox properties, which allow them to act as reducing agents, hydrogen donors and singlet oxygen quenchers (Rice-Evans et al., 1995; Sofidiya et al., 2006).

Anisopus mannii is a perennial herb with leaves spread and petiole 1.3 - 2 cm long, bearing a distinct gland at the apex, blade about 5.7 - 7.6 cm long and stem twining to a height of 3.7 - 4.6 m (Hutchinson and Dalziel, 1963). It is known as 'Kashe zaki' (Hausa) meaning "destroying sweetness". It is a familiar herb in the traditional medicinal preparations in northern Nigeria, where a decoction of the whole plant is used as a remedy for *diabetes*, diarrhea and pile. Previously, the proximate composition, mineral elements and anti-nutritional factors of *A. mannii* was reported (Aliyu et al., 2009). The phytochemical and antimicrobial screening of the stem aqueous extract (Sani et al., 2009) as well as the isolation of chemical constituents such as 1, 7-naphthyridine alkaloid- named anisopusin, 5α-hydroxy-lup-20(29)-en-3β-yl eicosanoate, [6]-gingerdione, [6]-dehydrogingerdione and ferulic acid from acetone extract of the stem bark have been reported (Tsopmo et al., 2009). The continued search among plant secondary metabolites for natural antioxidants has gained importance in recent years because of the increasing awareness of herbal remedies as potential sources of phenolic antioxidants. The aim of this work was to evaluate the *in vitro* antioxidant properties of the crude methanol extract and solvent fractions of *A. mannii*, in order to gain insight into the molecular basis for some of its therapeutic properties in folkloric medicine.

MATERIALS AND METHODS

Chemicals and reagents

Deionized water, Folin-Ciocalteu phenol reagent (Fluka, UK), gallic acid (Fluka, UK), 1, 1-diphenyl-2-picrylhydrazyl radical (DPPH) (Sigma-Aldrich Co.), Trichloroacetic acid (Sigma-Aldrich Co.), anhydrous ferric chloride, potassium ferricyanide, anhydrous sodium carbonate, Ascorbic acid (BDH Chemical Laboratory, England, UK) and all other chemicals were of analytical grade.

Plant material

A fresh plant of *A. mannii* (voucher No. 217) was collected in February 2007 at Dakace village along Jos road, Zaria, Nigeria. The plant was taxonomically authenticated and a specimen deposited at the Herbarium, Department of Biological Sciences, Ahmadu Bello University, Zaria, Nigeria.

Extraction and preliminary fractionation

Powdered leaf sample (250 g) was extracted exhaustively with methanol (maceration) for a week. The extract was filtered using Whatman filter paper no. 2 and concentrated on a Büchi rotary evaporator at 45°C giving the crude methanol extract (48.53 g). The fractionation pattern for isolating antioxidants as reported by Cho et al. (2003) was adapted. Forty grams (40 g) of crude methanolic extract was suspended in aqueous methanol (10%) and fractionated with chloroform (3 x 200 ml). The aqueous portion was extracted with ethyl acetate (3 x 200 ml) and *n*-butanol (3 x 200 ml) which afforded 1.25, 12.30 and 14.50 g of chloroform, ethyl acetate and *n*-butanol fractions, respectively. The fractionation flow chart is as shown in figure 1.

Phytochemical screening

Phytochemical screening of the crude methanol extract and solvent fractions for alkaloids, flavonoids, saponins, tannins, triterpenes, anthraquinones and cardiac glycosides were carried out to identify the constituents, using standard phytochemical methods as described by Sofowora (1993).

Evaluation of antioxidant activity

The determination of the free radical scavenging activity of the crude extract and solvent fractions were carried out using the DPPH (1, 1-diphenyl-2-picrylhydrazyl) assay as described by Mensor et al. (2001) with a slight modification. Various concentrations (250, 125, 50, 25 and 10 μgml^{-1}) of sample crude extract/ fractions in methanol were prepared. 1 ml of a 0.3 mM DPPH in methanol was added to 2.5 ml of the extracts or standard, and allowed to stand at room temperature in a dark chamber for 30 min. The change in colour from deep violet to light yellow was then measured at 518 nm on a spectrophotometer. The decrease in absorbance was then converted to percentage antioxidant activity (% AA) using the formula.

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$$

Blank = Methanol (1.0 ml) plus sample solution (2.0 ml); negative control = DPPH solution (1.0 ml, 0.25 mM) plus methanol 2.0 ml; ascorbic acid and gallic acid were used as standards.
The scavenging reaction between ($DPPH^{\bullet}$) and an antioxidant (H-A) can be written as:

$$(DPPH^{\bullet}) + (H\text{-}A) \rightarrow DPPH\text{-}H + (A^{\bullet})$$

Purple Yellow

Determination of total phenolic content

The total phenolic content of the crude methanol extract and ethyl acetate and n-butanol fractions were determined using the method of Mcdonald et al. (2001) with slight modifications. The calibration curve was prepared by mixing an ethanol solution of gallic acid (1 ml; 0.025 - 0.400 mg/ml) with 5 ml Folin-Ciocalteu reagent (diluted tenfold) and sodium carbonate (4 ml, 0.7 M). Absorbance values were measured at 765 nm and the standard curve was drawn. 1 ml of crude methanol extract (5 gL^{-1}) was also mixed with the above reagents and after 30 min the absorbance was measured to determine the total phenolic content. All determinations were carried out in triplicates. The total phenolics concentration in the extract in gallic acid equivalents (GAE) was calculated by using the following formula:

$$T = CV/M$$

Where T = total phenolic contents, milligram per gram plant extract,

Table 1. **Phytochemical screening of crude extract and fractions of *A. mannii.***

Phytochemicals	Crude methanol	Ethyl acetate	*n*-butanol
Alkaloids	+	+	-
Flavonoids	+	+	+
Saponins	+	+	+
Tannins	+	+	+
Triterpenes	+	+	+
Anthraquinones	-	-	-
Cardiac glycosides	+	-	+

+ = present, - = absent.

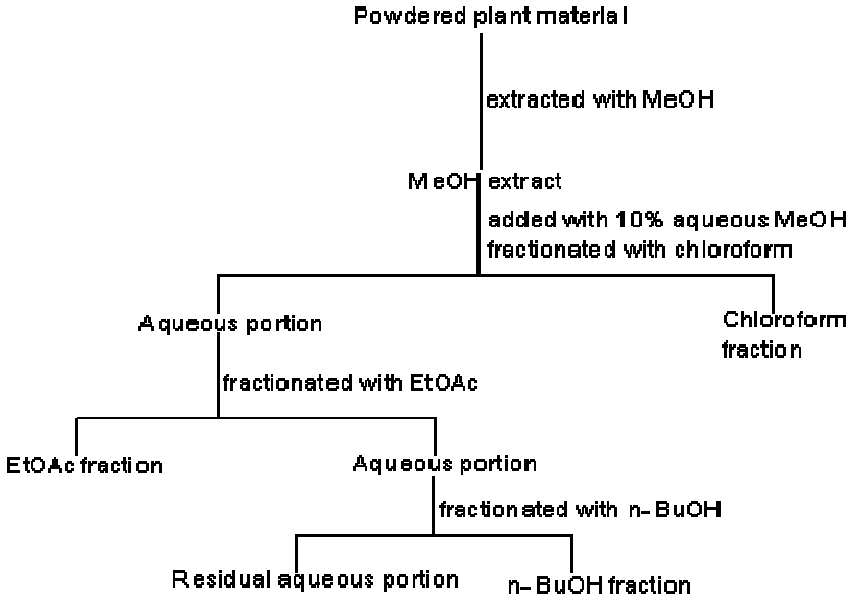

Figure 1. Extraction and fractionation of antioxidants from plant sample adapted from Cho et al. (2003).

in GAE; C = the concentration of gallic acid established from the calibration curve, milligram per milliliter (mg/ml); V = the volume of extract, milliliter; M = the weight of methanol plant extract, gram.

Reducing power assay

This was determined according to the method of Oyaizu (1986). The crude extract/fractions or standard (100 μgml^{-1}) was mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and potassium ferricyanide (1%, 2.5 ml). The mixture was incubated at 50°C for 20 min. Trichloroacetic acid (10%, 2.5 ml) was added to the mixture. A portion of the resulting mixture was mixed with $FeCl_3$ (0.1%, 0.5 ml) and the absorbance was measured at 700 nm in a spectrophotometer. The higher the absorbance of the reaction mixture, the higher the reducing power of the crude extract/fractions.

Statistical analysis

The experiments were carried out in triplicates. The results were given as mean ± standard deviation (SD). One way analysis of variance (ANOVA) was carried out to test for significant differences between the means of samples and standards. A difference was considered statistically significant when $p < 0.05$.

RESULTS

Phytochemical screening of the crude methanol extract, *n*-butanol and ethyl acetate fractions revealed the presence of saponins, triterpenes, flavonoids, tannins and alkaloids (Table 1). The results of the DPPH assay showed maximum percentage antioxidant activity at 250μgml^{-1} for the crude methanol extract, ethyl acetate and *n*-butanol fractions (Figure 2). The reducing powers at 100μgml^{-1} were 0.106, 0.110 and 0.112 nm for the crude ethanol extract, ethyl acetate and *n*-butanol fractions, respectively. The total phenolic concentration was highest in the ethyl acetate fraction; followed by *n*-butanol fraction and the crude methanol extract (Table 2).

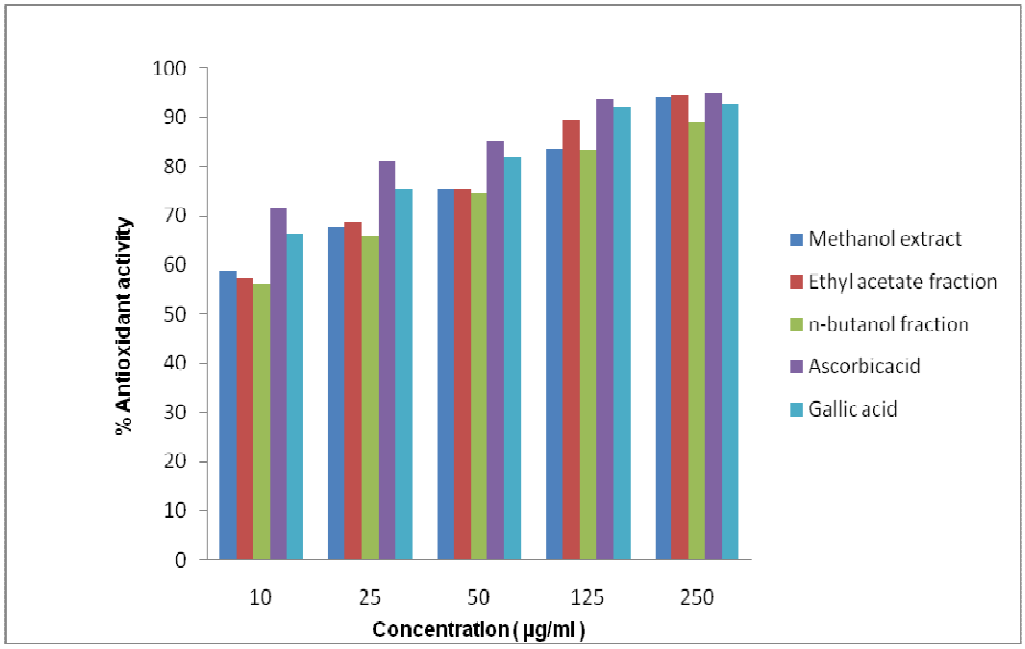

Figure 2. Antioxidant activity of *A. mannii* crude extract and fractions..

Table 2. Total phenolic content and Reducing power of *A. mannii* crude extract and fractions.

Extract/fractions	Total phenolic content (mg/g GAE)	Reducing power (nm)
Crude methanol	53.20	0.106 ± 0.01
Ethyl acetate	59.60	0.110 ± 0.02
n-butanol	56.40	0.112 ± 0.01
Gallic acid	-	0.096 ± 0.04

DISCUSSION

Phytochemicals, and especially polyphenols, have received increasing attention because of interesting new findings regarding their biological activities (Cho et al., 2003); they constitute a major group of compounds that act as primary antioxidants (Hatano et al., 1989). Phytochemicals such as flavonoids, saponins, tannins, triterpenes, cardiac glycosides and alkaloids were found to be present in the crude methanol extract/solvents fractions of *A. mannii*. The free radical scavenging activity of the DPPH assay of *A. mannii* showed maximum percentage antioxidant activity at 250 µgml^{-1} for crude methanol extract, ethyl acetate and *n*-butanol fractions. Figure 2 showed the relative increase in antioxidant activity of crude extract/fractions and standard samples; indicating a positive dose dependent radical reduction activity. The reduction mechanism of the DPPH radical correlates with the hydroxyl groups on the antioxidant molecule (Cotelle et al., 1996; Basnet et al., 1997), so the mechanism might involve the delocalization of an electron onto the *p*-substituted OH-group on the molecule prior to the donation of a second hydrogen to reduce DPPH$^{\bullet}$, which also depends on the stability and reaction potential of the molecular structure (Brand-Williams et al., 1995).

In the reducing power assay, presence of antioxidants in the extract reduced Fe^{3+}/ ferricyanide complex to the ferrous form. The reducing capacity of compounds could serve as indicator of potential antioxidant properties (Meir et al., 1995) and increased absorbance is proportional to the increased reducing power. The reducing power of the crude methanol extract and the ethyl acetate and n-butanol fractions which ranges from 0.106 - 0.112 nm are higher than that of standard gallic acid, but the differences were not significant ($P > 0.05$) (Table 2).The total phenolic content of the ethyl acetate fraction was highest and it corroborates the observed percent anti-oxidant activity, indicative of the involvement of phenolics in the radical scavenging activity of the ethyl acetate fraction. Generally, the crude extract and solvent fractions of *A. mannii* were found to possess high antioxidant activity.

Reactive oxygen species and associated free radicals have been implicated in the etiology of various human diseases including inflammation, metabolic disorders, cellular aging and atherosclerosis, heart disease, stroke,

Diabetes mellitus, cancer, malaria, rheumatoid arthritis and HIV/AIDS (Alho and Leinonen, 1999; Odukoya et al., 2005). Therefore, radical scavengers give promising indications of new therapeutic approaches (Cho et al., 2003). The significance of antioxidant evaluation of these extract and fractions lie in the structural requirement of the chemical constituents that could be linked to flavonoids, or tannins, which have been found present in the plant crude extract/fractions (Table 1). Many flavonoids have shown strong antioxidant properties (Raj and Shalini, 1999) and quercetin has been established as a strong antioxidant principle and is used as standard in antioxidant experiments (Thabrew et al., 1998).The capacity of plant extracts to act as reducing agents, hydrogen donors, singlet oxygen quenchers, metal chelators or free radical scavengers indicates their potential therapeutic properties for treating diseases related to free radical reactions.

In conclusion, the crude methanol extract and solvent fractions of *A. mannii* have indicated strong antioxidant activities at least, *in vitro.* The plant contained phenolic compounds which can serve as natural sources of antioxidants. The study highlights the significance of the free radical scavenging capacity and the potentials of *A. mannii* as a source of therapeutic agents. Further work should focus on the *in vivo* evaluations as well as the isolation, identification and spectral characterization of the radical scavenging components.

ACKNOWLEDGEMENTS

We are grateful to the authority of Ahmadu Bello University, Zaria, Nigeria, for providing the facilities for conducting the research work and also to Abdulkadir E. Ibrahim and Mubarak Balarabe Dambatta for the technical assistance.

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